PATENT APPLICATION

LIGHT-DRIVEN ENERGY GENERATION USING PROTEORHODOPSIN

INVENTORS

Edward F. DeLong and Oded Beja

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is cross-referenced to and claims priority from U.S Provisional application 60/201,602 filed 05/03/2000, which is hereby incorporated by reference.

STATEMENT REGARDING FEDERALLY SPONDORED RESEARCH OR DEVELOPMENT

This invention was supported in part by grant number OCE 0001619 from the National Science Foundation (NSF). The U.S. government has certain rights in the invention.

STATEMENT TO COMPUTER DISK AND SEQUENCE LISTING

This application includes a sequence listing of 65 sequences and a computer disk labeled "Sequence Listing for application entitled "Light-driven energy generation using proteorhodopsin" by Edward F. DeLong and Oded Beja" containing files "MBA101-SEQLIST.prj", dated "04/23/01" with 174,089 bytes, which is the PatentIn project file generated using PatentIn Version 3.0 software provided by the USPTO, and "MBA101-

SEQLIST.txt", dated "04/23/01" with 323,739 bytes, which is the generated sequence listing from the PatentIn project file MBA101-SEQLIST.prj using PatentIn Version 3.0 software, all which are herein incorporated. The information recorded in computer readable format on the incorporated computer disk labeled "Sequence Listing" containing files "MBA101-SEQLIST.prj" and "MBA101-SEQLIST.txt" are identical to the incorporated written sequence listing.

FIELD OF THE INVENTION

The present invention relates generally to gene expression of functional recombinant proteins in bacteria. More particularly, the present invention relates to proteorhodopsin genes and proteins that function as a light-driven energy generator in *Escherichia coli* (E. coli) and other bacteria.

BACKGROUND ART

Retinal (vitamin A aldehyde) is a chromophore that binds integral membrane proteins (opsins) to form light-absorbing pigments called rhodopsins. Rhodopsins are currently known to belong to two distinct protein families. The visual rhodopsins, found in the eye throughout the animal kingdom, are photosensory pigments. Archeal rhodopsins, found in extreme halophilic environments, function as light-driven protons pumps (bacteriorhodopsins), chloride ion pumps (halorhodopsins), or photosensory receptors (sensory rhodopsins). The two protein families show no significant sequence similarity and may have different origins. They do, however, share identical topologies characterized by seven transmembrane α–helices that form a pocket in which retinal is covalently linked, as a pronated Schiff base (helix G).

The archaeal rhodopsins are able to generate a photocycle which produces a chemiosmotic membrane potential in response to light, as such light energy is converted into biochemical energy. Recently, a protein with high sequence similarity to the archaeal rhodopsins has also been retrieved in the eukaryote Neurospora crassa (*J.A. Bieszke et al., Proceedings of National Academy of Sciences USA 96:8034, 1999*). The eucaryal rhodopsin formed a photochemically reactive pigment when bound to all-trans retinal and exhibited photocycle kinetics similar to those of archaeal sensory rhodopsins (*J.A. Bieszke et al., Biochemistry 38:14138, 1999*). To date, however, no rhodopsin-like sequences have been reported in members of the domain Bacteria, and no light-driven proton pumps based on rhodopsin have ever before been functionally expressed in *E coli.*

The phototropic conversion of light energy into biochemical energy using bacteriorhodopsin can be harnessed for a variety of processes and applications, such as bio-electronic applications and bio-materials, as has been reported in US Patent No. 5,757,525 for optical devices, US Patent No. 5,854,710 for optical Fourier processing, and US Patent No. 5,470,690 for optical information storage. Bacteriorhodopsin in bio-electronic applications is aimed to interface, integrate, or substitute the silicon based microelectronics systems as well as molecular devices. Bacteriorhodopsin as a bio-material is integrated, for instance, in optical films for light mediated computer memory applications and pattern recognition.

Prevsiously, archaeal rhodopsins capable of generating a chemiosmotic membrane potential in response to light had only been found in halophilic archaea. Therefore, rhodopsins that originate from archaea adapted to highly saline environments cannot be functionally expressed in *E. coli.* Finally, the isolation and cultivation of halorhodopsins is an elaborate

process. At present one does not foresee an economic utilization possible for this process (e.g. US Patent 5,290,699).

Accordingly, as one skilled in the art might readily acknowledge, there is a strong need to retrieve and provide rhodopsin-like sequences from naturally occurring members of the domain Bacteria.

OBJECTS AND ADVANTAGES

In light of the above, it is the primary objective of the present invention to provide rhodopsin-like sequences from naturally occurring members of the domain Bacteria. More specifically, it is the objective of the present invention to provide a method to retrieve proteorhodopsin genes from DNA of naturally occurring bacteria that encodes DNA sequence for proteorhodopsin proteins.

It is another objective of the present invention to provide proteorhodopsin-specific polymerase chain reaction primers that amplify the proteorhodopsin-containing gene from a DNA sample of naturally occurring bacteria.

It is yet another objective of the present invention to produce variants of a proteorhodopsin gene using the same proteorhodopsin-specific polymerase chain reaction primers by amplifying a proteorhodopsin-containing gene from of a mixed sample of naturally occurring bacteria.

It is still another objective of the present invention to provide an expression vector that produces a proteorhodopsin protein in *E. coli* and other bacteria.

It is another objective of the present invention to provide a light-driven energy generator in which the functional properties of proteorhodopsin are utilized. These properties include the ability to integrate within a host, for instance a cell membrane of *E. coli*, making an integrated proteorhodopsin protein, and the ability to bind retinal, making a light absorbing pigment.

It is another objective of the present invention to provide a light source and illuminate the light absorbing pigment to convert light energy into biochemical energy.

It is another objective of the present invention to provide a mediator and mediate the biochemical energy into electrical energy.

It is another objective of the present invention to provide methods to manipulate the kinetics of the light-driven energy generator.

The advantage of the present invention over the prior art is that it is not restricted to operate in halophilic archaea and could therefore be functionally expressed in *E. coli* and other bacteria. Accordingly, another advantage of the present invention is that it provides for a fast and cheap production method that allows for mass production of functionally active proteorhodopsin.

SUMMARY

The present invention provides proteorhodopsin gene and protein sequences retrieved from samples of naturally occurring members of the domain Bacteria. More specifically, the present invention provides a method for the retrieval and amplification of proteorhodopsin genes from DNA samples of naturally occurring marine bacteria. In accordance with several exemplary embodiments of the present invention, DNA samples were obtained from naturally occurring bacteria such as, for instance, marine proteobacteria, SAR86 bacteria, or recombinant DNA libraries containing naturally occurring bacteria. The present invention provides proteorhodopsin-specific polymerase chain reaction (PCR) primers to amplify a proteorhodopsin gene from DNA samples of these marine bacteria. The present invention also provides a device and method for the placement of proteorhodopsin genes in an expression vector to produce functional proteorhodopsin proteins in *E. coli* and other bacteria.

Accordingly, the present invention provides a method to produce and obtain variants of proteorhodopsin genes and proteins. The same proteorhodopsin-specific polymerase chain reaction primers amplify different variants of proteorhodopsin-containing genes from a mixed sample of naturally occurring bacteria. As one skilled in the art might readily acknowledge, these variants of a proteorhodopsin gene produce functional variations in the photocycle kinetics of the proteorhodopsin protein.

Furthermore, the present invention provides a light-driven energy generator that utilizes proteorhodopsin to convert light-energy into biochemical energy. This light-driven energy generator takes advantage of the functional properties of the proteorhodopsin protein once expressed in, for example, *E. coli* or other bacteria as is described in exemplary embodiments. These properties include the ability to integrate within a host such as, for instance, a cell

membrane of *E. coli* or other Bacteria, and thereby making an integrated proteorhodopsin protein or integrated cell membrane protein. These properties also include the ability to bind retinal and thereby making a light absorbing pigment. Illuminating the light absorbing pigment with a light source converts light energy into biochemical energy. Finally, the biochemical energy can be mediated into electrical energy by a mediator.

In accordance with exemplary embodiments, the present invention enables one skilled in the art to manipulate the kinetics of the proteorhodopsin protein photocycle once it is operational in the light-driven energy generator. In particular, the present invention provides examples in which the light source characteristics are manipulated. Examples are the manipulation of the delivery of fast-light pulses and/or the delivery of light at different wavelengths. The present invention also provides examples in which incremental additions of retinal influences the function of the light-driven energy generator. In addition, a proteorhodopsin gene or protein variant can be selected to determine an absorption spectra of the light absorbing pigment to change the kinetics of the light energy generator, for instance to meet a design/functional criteria of an application wherein proteorhodopsin is utilized.

BRIEF DESCRIPTION OF THE FIGURES

The objectives and advantages of the present invention will be understood by reading the following detailed description in conjunction with the drawings, in which:

FIG. 1 illustrates the phylogenetic tree of bacterial 16S rRNA gene sequences including that encoded on the 130 kb bacterioplankton BAC clone (EBAC31A8).

FIG. 2 provides a nucleotide sequence of polymerase chain reaction primer 1 (**Sequence ID No:2**) used to amplify a proteorhodopsin gene.

FIG. 3 provides a nucleotide sequence of polymerase chain reaction primer 2 (**Sequence ID No:3**) used to amplify a proteorhodopsin gene.

FIG. 4 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:4**) amplified from clone EBAC31A8 (**Sequence ID No:1**) using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence ID No:3**), and the deduced amino acid sequence (**Sequence ID No:5**) of the proteorhodopsin gene **Sequence ID No:4** amplified from clone EBAC31A8 (**Sequence ID No:1**).

FIG. 5 provides a map of the secondary structure of the proteorhodopsin protein (**Sequence ID No:7).** Single letter amino acid codes are used (according to J. Sasaki and J.L. Spudich, Biophys. J. 75:2435, 1998). Predicted retinal binding pocket residues are marked in black.

FIG. 6 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:8**) amplified from clone EBAC40E8 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:9**) of the proteorhodopsin gene **Sequence ID No:8** amplified from clone EBAC40E8.

FIG. 7 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:10**) amplified from clone EBAC41B4 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:11**) of the proteorhodopsin gene **Sequence ID No:7** amplified from clone EBAC41B4.

FIG. 8 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:12**) amplified from clone EBAC64A5 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID**

No:13) of the proteorhodopsin gene **Sequence ID No:12** amplified from clone EBAC64A5.

FIG. 9 provides a variants map of the DNA sequences of the proteorhodopsin gene with **Sequence ID No:4**, **Sequence ID No:8**, **Sequence ID No:10**, and **Sequence ID No:12** that were amplified from clone EBAC38A8, EBAC40E8, EBAC41B4 and EBAC64A5 respectively using the proteorhodopsin-specific PCR primer 1 (**Sequence ID No:2**) and 2 (**Sequence ID No:3**). Dots represent sequences having identical sequence as those in **Sequence ID No:4**.

FIG. 10 provides a variant map of the deduced amino acid sequences encoded by the proteorhodopsin gene with **Sequence ID No:4**, **Sequence ID No:8**, **Sequence ID No:10**, and **Sequence ID No:12** that were amplified from respectively EBAC38A8, EBAC40E8, EBAC41B4 and EBAC64A5 using the proteorhodopsin-specific primer 1 (**Sequence ID No:2**) and 2 (**Sequence ID No:3**). Lower case represents the PCR primer sequence region. Dots represent residues having identical sequence as those in **Sequence ID No:5**.

FIG. 11 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:14**) amplified from clone HOT0m1 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:15**) of the proteorhodopsin gene **Sequence ID No:14** amplified from clone HOT0m1.

FIG. 12 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:16**) amplified from clone HOT75m1 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:17**) of the proteorhodopsin gene **Sequence ID No:16** amplified from clone HOT75m1.

FIG. 13 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:18**) amplified from clone HOT75m3 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:19**) of the proteorhodopsin gene **Sequence ID No:18** amplified from clone HOT75m3.

FIG. 14 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:20**) amplified from clone HOT75m4 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:21**) of the proteorhodopsin gene **Sequence ID No:20** amplified from clone HOT75m4.

FIG. 15 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:22**) amplified from clone HOT75m8 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:23**) of the proteorhodopsin gene **Sequence ID No:22** amplified from clone HOT75m8.

FIG. 16 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:24**) amplified from clone MB0m1 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:25**) of the proteorhodopsin gene **Sequence ID No:24** amplified from clone MB0m1.

FIG. 17 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:26**) amplified from clone MB0m2 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:27**) of the proteorhodopsin gene **Sequence ID No:26** amplified from clone MB0m2.

FIG. 18 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:28**) amplified from clone MB20m2 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:29**) of the proteorhodopsin gene **Sequence ID No:28** amplified from clone MB20m2.

FIG. 19 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:30**) amplified from clone MB20m5 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:31**) of the proteorhodopsin gene **Sequence ID No:30** amplified from clone MB20m5.

FIG. 20 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:32**) amplified from clone MB20m12 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:33**) of the proteorhodopsin gene **Sequence ID No:32** amplified from clone MB20m12.

FIG. 21 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:34**) amplified from clone MB40m1 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:35**) of the proteorhodopsin gene **Sequence ID No:34** amplified from clone MB40m1.

FIG. 22 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:36**) amplified from clone MB40m5 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:37**) of the proteorhodopsin gene **Sequence ID No:36** amplified from clone MB40m5.

FIG. 23 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:38**) amplified from clone MB40m12 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:39**) of the proteorhodopsin gene **Sequence ID No:38** amplified from clone MB40m12.

FIG. 24 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:40**) amplified from clone MB100m5 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:41**) of the proteorhodopsin gene **Sequence ID No:40** amplified from clone MB100m5.

FIG. 25 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:42**) amplified from clone MB100m7 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:43**) of the proteorhodopsin gene **Sequence ID No:42** amplified from clone MB100m7.

FIG. 26 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:44**) amplified from clone MB100m9 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:45**) of the proteorhodopsin gene **Sequence ID No:44** amplified from clone MB100m9.

FIG. 27 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:46**) amplified from clone MB100m10 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:47**) of the proteorhodopsin gene **Sequence ID No:46** amplified from clone MB100m10.

FIG. 28 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:48**) amplified from clone PALB1 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:49**) of the proteorhodopsin gene **Sequence ID No:48** amplified from clone PALB1.

FIG. 29 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:50**) amplified from clone PALB2 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:51**) of the proteorhodopsin gene **Sequence ID No:50** amplified from clone PALB2.

FIG. 30 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:52**) amplified from clone PALB5 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:53**) of the proteorhodopsin gene **Sequence ID No:52** amplified from clone PALB5.

FIG. 31 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:54**) amplified from clone PALB7 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:55**) of the proteorhodopsin gene **Sequence ID No:54** amplified from clone PALB7.

FIG. 32 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:56**) amplified from clone PALB6 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:57**) of the proteorhodopsin gene **Sequence ID No:56** amplified from clone PALB6.

FIG. 33 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:58**) amplified from clone PALB8 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:59**) of the proteorhodopsin gene **Sequence ID No:58** amplified from clone PALB8.

FIG. 34 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:60**) amplified from clone PALE1 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:61**) of the proteorhodopsin gene **Sequence ID No:60** amplified from clone PALE1.

FIG. 35 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:62**) amplified from clone PALE6 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:63**) of the proteorhodopsin gene **Sequence ID No:62** amplified from clone PALE6.

FIG. 36 provides the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:64**) amplified from clone PALE7 using PCR primers 1 (**Sequence ID No:2**) and 2 (**Sequence No:3**), and the deduced amino acid sequence (**Sequence ID No:65**) of the proteorhodopsin gene **Sequence ID No:64** amplified from PALE7.

FIG. 37 illustrates a phylogenetic tree of different proteorhodopsin genes.

FIG. 38 provides an example of an alignment of proteorhodopsin amino acid sequences.

FIG. 39 provides a light-driven energy generator that utilizes proteorhodopsin.

FIG. 40 provides an example of a proteorhodopsin-expressing *E. coli* cell suspension (+) compared to control cells (-), both with all-*trans* retinal.

FIG. 41 provides an example of absorption spectra of retinal-constituted proteorhodopsin in E coli membranes and a negative control.

FIG. 42 provides an example of a light-driven transport of protons by a proteorhodopsin–expressing E. coli cell suspension.

FIG. 43 provides an example of a transport of [^{3}H]TPP$^+$ in E. coli right-side-out vesicles containing expressed proteorhodopsin, reconstituted with or without 10 μM retinal in the presence of light or in the dark.

FIG. 44 provides an example of laser flash-induced absorbance changes in suspensions of *E. coli* membranes containing proteorhodopsin.

FIG. 45 provides an example of absorption spectra of retinal-constituted proteorhodopsin in *E. coli* membranes.

DETAILED DESCRIPTION

Although the following detailed description contains many specifics for the purposes of illustration, anyone of ordinary skill in the art will appreciate that many variations and alterations to the following details are within the scope of the invention. Accordingly, the following preferred embodiment of the invention is set forth without any loss of generality to, and without imposing limitations upon, the claimed invention.

Proteorhodopsin

The present invention provides rhodopsin-like gene and protein sequences retrieved from naturally occurring members of the domain Bacteria. More specifically, the present invention provides a method for the retrieval and amplification of proteorhodopsin genes from DNA samples of naturally occurring marine bacteria. In accordance with exemplary embodiments of the present invention, DNA samples were obtained from naturally occurring marine bacteria such as bacteria from the SAR86 group. Provided as an exemplary embodiment of the SAR86 group, DNA samples were obtained from a bacterioplankton Bacterial Artificial

Chromosome (BAC) clone BAC31A8 (also referred to as EBAC31A08). In general, as will be appreciated by those of ordinary skill in the art, suitable DNA samples can also be obtained from other sources, e.g., from a marine environment or from a recombinant DNA library containing genomic fragments of samples of naturally occurring bacteria.

FIG. 1 shows the phylogenetic tree of bacterial 16S rRNA gene sequences including that encoded on the EBAC31A8. **FIG. 1** also shows the relationship of EBAC31A8 to the SAR86 bacteria group as well as to the gamma-proteobacteria group. A subclone shotgun library was constructed from BAC clone 31A8, and subclones were sequenced in both directions on the MegaBACE 1000 capillary array electrophoresis DNA sequencing instrument (Molecular Dynamics, Sunnyvale, CA). Sequence analysis of a 130-kb genomic DNA that encodes the ribosomal RNA operon from BAC31A8, reveals an open reading frame encoding a proteorhodopsin. In an exemplary embodiment, the contiguous sequence was assembled using SEQUENCHER 3.1.1 software (Gene Codes Co., Ann Arbor, MI). Other sequencing techniques can also be used, as will be recognized by those skilled in the art. The sequence of the proteorhodopsin-containing contig has been deposited in GenBank under accession #AF279106 and deposit date October 23rd, 2000. **Appendix A**, hereby incorporated, shows the nucleotide sequence of the BAC clone BAC31A8 (**Sequence ID No:1**) which contains the 130 kilobases genomic DNA from a naturally occurring marine bacterium.

Proteorhodopsin was amplified from the 130 kilobase bacterioplankton BAC clone 31A8 (**Sequence ID No:1**) by polymerase chain reaction (PCR), using the proteorhodopsin-specific primers 5'-aCCATGGgtaaattattactgatattagg-3' (**Sequence ID No:2** and shown in **FIG. 2**) and 5'-agcattagaagattctttaacagc-3' (**Sequence ID No:3** and shown in **FIG. 3**). References for PCR are, for instance, *The Polymerase Chain Reaction,* Mullis et al., Ed. (Birkhauser, Boston, 1994) and U.S. Patent Nos. 4,683,195 and 4,683,202 to Mullis et al. The proteorhodopsin-

specific PCR primers include the addition of 3 nucleotides that encoded one amino acid not found in the native gene sequence of clone BAC31A8 (**Sequence ID No:6**), in the second amino acid position which is a glycine located on the 2nd codon ("GGT"). Therefore, compare the second amino acid position in the **Sequence ID No:5** using PCR primers 1 and 2 with the native **Sequence ID no:7**. This addition of one non-native amino acid created a new restriction endonuclease site (NcoI site) not present in the native sequence. This allowed subcloning of the amplified fragment into the NcoI restriction site of an expression vector pBAD TOPO TA Cloning® Kit (Invitrogen, La Jolla, CA). The present invention is not limited to the use of this type of expression vector and other expression vectors could also be used.

FIG. 4 shows the nucleotide sequence of the proteorhodopsin gene (**Sequence ID No:4**) that results from amplification of the proteorhodopsin-containing DNA in BAC31A8 using proteorhodopsin-specific PCR primers **Sequence ID No:2** and **Sequence No:3**. **FIG. 4** also shows the deduced amino acid sequences (**Sequence ID No:5**) encoded by the proteorhodopsin gene (**Sequence ID No:4**).

FIG. 5 shows an exemplary embodiment of a secondary structure of proteorhodopsin after it has been folded in a cell membrane **510** and bonded with retinal **520**. **FIG. 5** shows the native proteorhodopsin gene (**Sequence ID No:6**) obtained from clone BAC31A8 and encodes a proteorhodopsin protein of 249 amino acids with a molecular weight of 27 kD (**Sequence ID No:7**). In **FIG. 5, 530** indicates seven transmembrane domains, a typical feature of the rhodopsin protein family, that aligned well with the corresponding helices of the archaeal rhodopsins. **FIG. 5** also shows the amino acid residues that form a retinal binding pocket indicated by **520**. Although the proteorhodopsin proteins shown in **FIGS. 4** and **5** both originate from BAC31A8, they differ with respect to the second amino acid position.

The reason is that the proteorhodopsin-specific PCR primers that were used to amplify the proteorhodopsin gene from BAC31A8 (which resulted in proteorhodopsin protein as in **FIG. 4**; **Sequence ID No:5**) included the addition of 3 nucleotides. These 3 nucleotides encoded one amino acid not found in the native gene sequence (**Sequence ID No:6**), in the second amino acid position which is a glycine located on the 2nd codon ("GGT"). Proteorhodopsin protein (**Sequence ID No:7**) as shown in **FIG. 5** originates from the native gene sequence without the addition of the 3 nucleotides. As mentioned above, the addition of the 3 nucleotides created a new restriction endonuclease site (NcoI site) that was not present in the native sequence and thereby allowed the amplified fragment to be subcloned into the NcoI site of the expression vector.

In the exemplary embodiment presented above, PCR primers with **Sequence ID No:2** and **Sequence ID No:3** were used. In general, the present invention provides a method for designing different proteorhodopsin-specific PCR primers that are all capable of amplifying a proteorhodopsin gene from DNA samples of naturally occurring microbial populations by polymerase chain reaction. In designing these primers one first needs to determine a DNA sequence of a proteorhodopsin gene. Then one can design oligodeoxynucleotide primers with a Watson-Crick base pair complementary to 5' and 3' ends of the proteorhodopsin gene.

Variants of Proteorhodopsin

In the previous section, an exemplary embodiment is provided of a proteorhodopsin gene and protein. The present invention also provides the retrieval of genetic variations of proteorhodopsin from naturally occurring genetic variations in naturally occurring bacterial populations. These genetic variations in proteorhodopsin sequences result in functional variations in the proteorhodopsin proteins as is discussed below.

The present invention enables one skilled in the art to use the same proteorhodopsin-specific PCR primers as shown in **FIGS. 2** and **3** to successfully amplify different sequence variants from DNA originating from mixed naturally occurring bacterial populations when it is compared to for instance the proteorhodopsin gene as shown in **FIG. 4**. As mentioned above, different proteorhodopsin-specific PCR primers could be used to amplify genetic variants of proteorhodopsin.

FIGS. 6-8 show exemplary embodiments of three different and unique variants of the proteorhodopsin gene that were retrieved from a recombinant DNA library of other naturally occurring bacteria (i.e. the bacterial artificial chromosome library (BAC)). In general, genetic variants could be obtained from different DNA libraries containing naturally occurring bacteria as well as from samples of naturally occurring bacteria. **FIG. 6** shows the variant of the proteorhodopsin gene sequence (**Sequence ID No:8**) that is amplified from the BAC clone 40 (BAC40E8) with the same proteorhodopsin-specific PCR primers as provided in **Sequence ID No:2** and **3**. Accordingly, **FIG. 6** also shows the deduced amino acid sequence (**Sequence ID No:9**) of the genetic variant of proteorhodopsin shown in **FIG. 6**. **FIG. 7** shows the variant of the proteorhodopsin gene sequence (**Sequence ID No:10**) that is amplified from the BAC clone 41 (BAC41B4) with the same proteorhodopsin-specific PCR primers as provided in **Sequence ID No:2** and **3**. Accordingly, **FIG. 7** also shows the deduced amino acid sequence (**Sequence ID No:11**) of the genetic variant of proteorhodopsin shown in **FIG. 7**. **FIG. 8** shows the variant of the proteorhodopsin gene sequence (**Sequence ID No:12**) that is amplified from the BAC clone 64 (BAC64A5) with the same proteorhodopsin-specific PCR primers as provided in **Sequence ID No:2** and **3**. Accordingly, **FIG. 8** also shows the deduced amino acid sequence (**Sequence ID No:13**) of the genetic variant of proteorhodopsin shown in **FIG. 8**.

FIG. 9 provides a variants map of the nucleotide sequences of the proteorhodopsin gene **Sequence ID No:4**, **Sequence ID No:8**, **Sequence ID No:10**, and **Sequence ID No:12** amplified from respectively BAC31A8, BAC40E8, BAC41B4 and BAC64A5 using the proteorhodopsin-specific PCR primers **Sequence ID No:2** and **Sequence ID No:3**. In **FIG. 9** lower case letters represent the PCR primer sequence region. Dots represent residues having identical sequence as those in **Sequence ID No:4**. These proteorhodopsin gene sequences differ by as much as 31 nucleotides as is shown in **FIG. 10**. **FIG. 10** provides a variant map of the deduced amino acid sequences of the proteorhodopsin genes shown in **FIG. 9**.

Using the same proteorhodopsin-specific PCR primers, as for instance shown in **FIGS. 2** and **3**, proteorhodopsin genes were also amplified from bacterioplankton extracts. As mentioned above, any proteorhodopsin-specific PCR primer can be used. These bacterioplankton extracts include those from the Monterey Bay (referred to as MB clones), the Southern Ocean (Palmer Station, referred to as PAL clones), and waters of the central North Pacific Ocean (Hawaii Ocean Time series station, referred to as HOT clones).

FIGS. 11-36 show exemplary embodiments of different and unique variants of proteorhodopsin that were retrieved from the MB clones, PAL clones, and HOT clones. **FIGS. 11-36** each show a variant of a proteorhodopsin gene sequence that is amplified with the same proteorhodopsin-specific PCR primers as provided in **Sequence ID No:2** and **Sequence ID No:3** from respectively clones HOT0m1, HOT75m1, HOT75m3, HOT75m4, HOT75m8, MB0m1, MB0m2, MB20m2, MB20m5, MB20m12, MB40m1, MB40m5, MB40m12, MB100m5, MB100m7, MB100m9, MB100m10, PALB1, PALB2, PALB5, PALB7, PALB6, PALB8, PALE1, PALE6 and PALE7. The proteorhodopsin gene sequences retrieved from clones HOT0m1, HOT75m1, HOT75m3, HOT75m4, HOT75m8, MB0m1, MB0m2, MB20m2, MB20m5, MB20m12, MB40m1, MB40m5, MB40m12, MB100m5,

MB100m7, MB100m9, MB100m10, PALB1, PALB2, PALB5, PALB7, PALB6, PALB8, PALE1, PALE6 and PALE7, have respectively **Sequence ID Nos: 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62,** and **64**. Accordingly, **FIGS. 11-36** also show the deduced amino acid sequence of each genetic variant of proteorhodopsin. The deduced amino acid sequence encoded by the proteorhodopsin gene retrieved from clones HOT0m1, HOT75m1, HOT75m3, HOT75m4, HOT75m8, MB0m1, MB0m2, MB20m2, MB20m5, MB20m12, MB40m1, MB40m5, MB40m12, MB100m5, MB100m7, MB100m9, MB100m10, PALB1, PALB2, PALB5, PALB7, PALB6, PALB8, PALE1, PALE6 and PALE7, have respectively **Sequence ID Nos: 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63,** and **65**.

In an exemplary embodiment shown in **FIG. 37**, fifteen different variants of proteorhodopsin in the PCR generated MB gene library **3710** were detected, falling into three clusters. The MB gene library includes MB clones MB0m2, MB40m5, MB20m2, MB40m12, MB100m10, MB20m12, MB40m1, MB100m5, MB20m5, MB100m7, MB0m1, and MB100m9 as well as BAC clones BAC40E8, BAC31A8 and BAC64A5. **FIG. 37** is based on a phylogenetic analysis of the inferred amino acids of cloned proteorhodopsin genes. Evolutionary distances calculated from 220 positions were used to infer the tree topology by the neighbor joining method using the PaupSearch program of the Wisconsin Package version 10.0 (Genetics Computer Group (GCG), Madison Wisconsin). Other methods could also be used. The variants of the MB library share at least 97% identity over 248 amino acids, as shown in **FIG. 38**, and 93% identity at the DNA level. All the PCR amplified proteorhodopsin genes from Antarctic marine bacterioplankton (e.g. the PAL clones) were different from those of Monterey Bay (e.g. the MB clones) sharing 78% identity over 248 amino acids with the Monterey clade. The changes in amino acid sequences were not restricted to the hydrophilic loops, but spread over the entire protein including changes near the retinal binding domain

3830 as shown in **FIG. 38**, which are predicted retinal-binding residues. **FIG. 38** shows an example of a multiple alignment of proteorhodopsin amino acid sequences that were obtained from different clones **3820.** The secondary structure is derived from hydropathy plots (boxes **3810** shows trans-membrane helices).

Light-driven energy generator

FIG. 39 provides a light-driven energy generator **3900** that utilizes proteorhodopsin, as obtained from naturally occurring bacteria as described above, to convert light-energy into biochemical energy. Light-driven energy generator **3900** takes advantage of the functional properties of the proteorhodopsin protein once expressed in, for instance, *E. coli* and other bacteria. These properties include the ability of proteorhodopsin **3906** to integrate within the cell membrane **3904** of, for instance, *E. coli* making an integrated proteorhodopsin protein **3908** (also called an integrated cell membrane protein). These properties also include the ability of proteorhodopsin **3906** to bind retinal **3910**, making a light absorbing pigment **3912**. The source of retinal **3910** is not limited to chromophore retinal but could also include chemical derivatives of retinal, such as 3-methyl-5-(1-pyryl)-2E,4E-pentadienal, 3,7-dimethyl-9-(1-pyryl)-2E,4E,6E,8E-nonatetraenal, all-trans-9-(4-azido-2,3,5,6-tetrafluorophenyl)-3,7-dimethyl-2,4,6,8,-nonatetraenal and 2,3-dehydro-4-oxoretinal. Illuminating light absorbing pigment **3912** with a light source **3914** results in a chemiosmotic gradient or proton pump in which light energy **3916** is converted into biochemical energy **3918**. The chemiosmotic gradient involves pumping of protons from the inside to the outside of cell membrane **3904**. When the protons return to the inside of cell membrane **3904** it produces biochemical energy **3918** via a proton translocating ATP-ase. Finally, the biochemical energy **3918** is harnessed by a mediator **3920** to produce energy **3922** for a particular process. For example, since proteorhodopsin functions as a light driven proton pump, it generates energy in the form of a proton motive force across the host cell membrane

upon illumination. This light-driven proton motive force can be converted to many other forms of energy, one example above being the regeneration of adenosine triphosphate (ATP), via a proton-translocating ATPase. This coupling of the proton motive force generated by proteorhodopsin, for use by proton-translocating ATPases to synthesize ATP, could be accomplished both in living cells, as well as in artificially constructed membrane systems such as liposomes. Proteorhodopsin-based systems can convert light energy to a wide variety of useful mechanical, chemical, and electrical energy forms, for many industrial and technological applications. These include, but are not limited to, use in targeted drug delivery, uses as primary or secondary energy generators for biocatalyic reactors, fuel cells and nano-machines (including molecular motors), as well as uses in molecular switching or data storage devices.

Applications that can potentially benefit from proteorhodopsin-light driven energy generation are, for instance, bio-electronics applications that are aimed to interface, integrate, or substitute the silicon based microelectronics systems as well as molecular devices. Other applications that can potentially benefit from proteorhodopsin-light driven energy generation are, for instance, in bio-materials, wherein proteorhodopsin is integrated as a bio-material in, for instance, optical films for light mediated computer memory applications, optical information storage and pattern recognition.

Alternatively, proteorhodopsin is useful for a process to enhance yield or increase the potential of recombinant protein production or converting the light induced membrane potential into cellular signals, including modulation of gene expression. The biochemical energy derived from functional proteorhodopsin exposed to light could be harnessed to support a variety of cellular processes. For instance, the energy derived from light-mediated proton pumping could be used to enhance the production of secondary metabolites, or

recombinant proteins in host cells, such as *E. coli.* Often, production of specific compounds in the biotechnology industry is limited, since their optimal expression or production occurs in the late stationary phase of growth, when energy reserves of the host cells are low. Retinal-bound proteorhodopsin expressed in such cells would provide an ample source of biochemical energy, by simple illumination. Proteorhodopsin-mediated light driven proton production could enhance any variety of biosynthetic or physiological processes which require energy.

The biochemical energy derived from proteorhodopsin light driven proton pumping could also be converted to other generally useful energy forms, for example electricity. Microbial fuel cells currently use carbon-based compounds, such as glucose, as the primary energy source. Via specific mediators of reduction potential (e.g. electrons), these microbial fuel cells convert cellular biochemical energy to electrical potential. Unlike carbon-based microbial fuel cells, proteorhodopsin uses light as the energy source, that can then be converted into a chemiosmotic potential, and finally into cellular biochemical energy by membrane-bound proton ATP-ases. Therefore, the use of proteorhodopsin could be employed to derive energy from light as the primary or supplementary energy source, that could then be converted into electrical potential (analogous microbial fuel cells that derive their energy from glucose).

In addition to energy generation in vivo in living cells, membranes containing proteorhodopsin could be used to enhance or enable other specific processes in vitro. Polymers produced from proteorhodopsin-containing membranes may have specific properties that could be used similarly to those containing bacteriorhodopsin. One example includes the use of these light sensitive molecules for optical computing applications.

As shown in **FIG. 39**, the kinetics of proteorhodopsin as it is utilized in **3900** is influenced by various factors such as the type of light source **3914** and the manipulation of light source **3914** in terms of frequency and/or wavelength at which the light **3916** is delivered. Light source **3914** could be any type of light source that delivers light energy **3916** that would be absorbed by light absorbing pigment **3918**. For example, the light source **3914** could be tuned to optimally excite rhodopsin variances with an absorbance maximum of 490 nm or alternatively those rhodopsins with an absorbance maximum of 520 nm. Manipulation of the light source **3914** or the light **3916** being emitted by the light source **3914,** for example, involves changing the frequency of fast-light pulses or the delivery of light **3916** as individual pulses, a train of pulses, or a continuous source of light. Manipulation also involves changing the wavelength of the delivery of light **3916** at different wavelengths. In addition, as is clear for one skilled in the art, changing the frequency and/or amount of retinal that will bind within integrated cell membrane protein **3908** also varies the function of proteorhodopsin. Finally, as was mentioned in the previous section, genetic variants of proteorhodopsin result in variants of the proteorhodopsin proteins that changes the kinetics of **3600** due to a difference in absorption of light at different wavelengths. The functional expression of such variation in these proteorhodopsin proteins adds another source of variation to the kinetics of proteorhodopsin as it is utilized in **3900**.

As shown in **FIG. 39**, the light-driven energy generator includes a host **3902**. In the present invention, as a preferred embodiment, host **3902** is a cell membrane preparation of *E. coli*. However, the present invention is not limited to the use of *E. coli* and, alternatively, other bacteria or eukaryotes could be used to provide host **3902** as an intact cell (in vivo) and/or as a cell membrane preparation (in vitro). For example, but not limited to, bacteria and yeast with developed genetic systems such as Bacillus spp. Species, Saccharomyces spp., Streptomyces spp. or Pichia spp. could be used as host for the expression of proteorhodopsin.

In addition, in case a cell membrane preparation (in vitro) is used, host **3902** becomes equivalent to cell membrane **3904**.

The light-driven energy generator **3900**, as shown in **FIG. 39**, further includes proteorhodopsin **3906**. Proteorhodopsin is presented in the form of the earlier presented expression vector containing a proteorhodopsin gene or one of its variants. Once proteorhodopsin **3906** has been put into host **3902**, the proteorhodopsin expression vector expresses the proteorhodopsin protein in host **3902**. An integral cell membrane protein **3908** is created in which the proteorhodopsin protein inserts into and folds properly within the cell membrane **3904**. This is accomplished in the *E. coli* host by virtue of the native signal sequence found in the 5' end of the proteorhodopsin gene. It could also be accomplished by replacement of native sequence with another host-specific signal sequence in non-*E. coli* host systems.

As shown in **FIG. 39**, once retinal **3910** is added to cell membrane **3904**, retinal **3910** binds within integrated cell membrane protein **3908** and forms a light absorbing pigment **3912**. The particular example of **FIG. 40** shows an integrated proteorhodopsin protein **3908** bound to retinal **3910** in *E. coli*. Chemical derivatives of retinal (as discussed above) could also be used as a substitute chromophore to generate functional proteorhodopsin. For the particular example of **FIG. 40**, the proteorhodopsin protein was cloned with its native signal sequence and included an addition of the V5 epitope, and a polyhistidine tail in the C-terminus. The proteorhodopsin protein was expressed in host **3902**, i.e. *E. coli* outer-membrane protease-deficient strain UT5600, and induced with 0.2 % arabinose for 3 hours. Cell membranes **3904** were prepared and resuspended in 50 mM Tris-Cl (pH 8.0) and 5 mM $MgCl_2$. **FIG. 40** shows a proteorhodopsin-expressing *E.coli* cell suspension. After 3 hours of induction in the presence of 10 μM all-trans retinal, cells expressing the protein acquire a reddish

pigmentation as indicated by **4010** and the + (plus) symbol. **FIG. 40** also shows that a cell suspension using the same PCR primers (**Sequence ID No:2** and **3**) but now in opposite orientation as a negative control, did not acquire a reddish pigmentation as indicated by **4020** and the – (minus) symbol.

FIG. 41 shows an exemplary embodiment of the absorption spectra of light absorbing pigment **3912** upon illumination with light source **3914** as is shown in **FIG 39**. As mentioned above, the light absorbing pigment is a retinal-reconstituted proteorhodopsin in *E. coli.* **FIG. 41** shows absorption spectra of light absorbing pigment **3912** as well as a negative control. After retinal **3910** addition to integrated proteorhodopsin protein **3908**, light absorbing pigment **3912** was made. The retinal **3910** addition was done at selected time points, i.e. 10, 20, 30 and 40 min, and shows a progression from low to high absorption values indicated by respectively **4110**, **4120**, **4130** and **4140** upon illumination with light source **3914**. **FIG. 41** also shows the absorption spectra of retinal **3910** addition at these similar time points but now to a negative control of retinal **3910** containing a proteorhodopsin **3906** that was created using the same PCR primers in opposite orientation. **4150**, **4160**, **4170** and **4180** indicate the four absorption spectra for the negative control. An absorption peak at 520 nm was observed after 10 minutes (**4110**) of incubation as illustrated in **FIG. 41**. On further addition of retinal, the peak at 520 nm increased, and had a ~100 nm half bandwidth. The 520 nm absorption peak was generated only in membranes containing proteorhodopsin **3906**, and only in the presence of retinal **3910**. The red shifted λmax of retinal (λmax = 370 nm in the free state) is indicative of a protonated Shiff base covalent linkage of retinal to proteorhodopsin.

FIG. 42 shows an exemplary embodiment of the light mediated proton pump of the light-driven energy generator **3900** indicating the conversion of light energy **3916** as shown in **FIG. 39**. The proton pump action is illustrated by measuring pH changes in the medium

surrounding the host **3902**, which in this particular example involves a cell suspension of *E. coli*, illuminated by light source **3914**. The beginning and cessation of illumination (with yellow light >485 nm delivered by **3916**) is indicated **4110** ("ON") and **4120** ("OFF") respectively. The cells were suspended in 10 mM NaCl, 10 mM $MgSO_4 \cdot 7H_2O$ and 100 μM $CaCl_2$. Net outward transport of protons was observed solely in proteorhodopsin-containing *E. coli* cells, and only in the presence of retinal **3910** and light **3916** and is indicated by **4210** in **FIG. 42**. Light-induced acidification of the medium was completely abolished by the presence of 10 μM of the protonophore CCCP.

FIG. 43 is an exemplary embodiment showing that illumination by light source **3914** generates an electrical potential at the membrane **3904** in proteorhodopsin-containing right-side-out membrane vesicles, in the presence of retinal **3910**, reaching –90 mV after 2 minutes from light **3916** onset. Transport of $[^3H]TPP^+$ in *E. coli* right-side-out vesicles containing expressed proteorhodopsin, reconstituted with (**4310** and **4320**) or without (**4330** and **4340**) 10 μM retinal **3910** in the presence of light (**4310** and **4330**) delivered by the light source **3914** or in the dark (**4320** and **4340**). **FIG. 43** shows that proteorhodopsin, in its form of **3912** as a light absorbing pigment, pumps protons from the inside to the outside of cell membrane in a physiologically relevant range. The ability of proteorhodopsin to generate a physiologically significant membrane potential, even when heterologously expressed in nonnative membranes, is consistent with the proton pumping function for proteorhodopsin in the native gamma proteobacteria from which it is derived.

FIG. 44 is an exemplary embodiment showing that proteorhodopsin can have a fast photocycle and can therefore be characterized as a fast and therefore efficient transporter of protons. For the particular example of **FIG. 44**, light absorbing pigment **3912** is induced by laser pulses delivered by light source **3914**. Laser pulse-induced absorption changes are

shown by **3912** in host **3902**, which in this case are suspensions of *E. coli* membranes containing proteorhodopsin. A 532-nm pulse (6 ns duration, 40 mJ) was delivered at time 0 and absorption changes were monitored at various wavelengths in the visible range in a lab-constructed pulse photolysis system. 64 transients were collected for each wavelength. **4410** indicates transients at 3 wavelengths exhibiting maximal amplitudes. **4420** indicates absorption difference absorption spectra calculated from amplitudes at 0.5 ms (indicated by **4430**) and between 0.5 ms and 5.0 ms (indicated by **4440**). In **4410**, transient depletion occurred near the absorption maximum of pigment **3912** (500-nm trace indicated by **4450**), and transient absorption increase was detected at 400 nm (indicated by **4460**) and 590 nm (indicated by **4470**), indicating a functional photocyclic reaction pathway. In **4420**, the absorption difference spectrum shows that within 0.5 ms an intermediate with maximal absorption near 400 nm is produced (indicated by **4430**), typical of unprotonated Schiff base forms (M intermediates) of retinylidene pigments. The 5-ms minus 0.5-ms difference spectrum **4440** shows that following M decay an intermediate species red-shifted from the unphotolyzed 520-nm state appears. The decay of proteorhodopsin final intermediate is the rate limiting step in the photocycle and is fit well by a single exponential process of 15 ms, with an upward baseline shift of 13% of the initial amplitude.

As mentioned above, a proteorhodopsin gene or protein variant can be selected to determine an absorption spectra of the light absorbing pigment to change the kinetics of the light energy generator **3900**, for instance to meet a design/functional criteria of an application wherein proteorhodopsin is utilized. **FIG. 45** shows an exemplary embodiment of different absorption spectra of retinal-reconstituted proteorhodopsins in *E. coli* as a function of wavelength **4510**. As shown in **FIG. 45**, the absorbance **4520** is different and depends on the clone from which the proteorhodopsin was amplified. In this particular example, 5 μm all-*trans* retinal was added to the membranes suspensions in a 100 mM phosphate buffer, with a pH 7.0, and

absorption spectra were recorded. The four spectra **4530, 4540, 4550,** and **4560** are respectively for the proteorhodopsin genes retrieved from clones HOT75m4, PALE6, HOT0m1, and BAC31A8 at 1 hour after retinal addition. The proteorhodopsin gene retrieved from clone HOT75m4 **4530** and PALE6 **4540** produced a blue (490 nm) absorption maximum. The proteorhodopsin gene retrieved from clone HOT0m1 **4550** and BAC31A8 **4560** produced a green (527 nm) absorption maximum. In general, a range of wavelengths could be obtained that is not limited to the range shown in the example of **FIG. 45.**

It will be clear to one skilled in the art that the above embodiment may be altered in many ways without departing from the scope of the invention, such as for instance by mutagenesis to change the genetic sequence of proteorhodopsin and thereby changing the kinetics of the proteorhodopsin protein once it is expressed. Accordingly, the following claims and their legal equivalents should determine the scope of the invention.